III.-RESULTADOS

Primer trabajo

“Differential involvement of phosphatidylinositol 3-kinase and p42/p44 mitogen protein kinase pathways in brain-derived neurotrophic factor-induced trophic effects on cultured striatal neurons”

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Differential involvement of phosphatidylinositol 3-kinase and p42/p44 mitogen activated protein kinase pathways in brain-derived neurotrophic factor-induced trophic effects on cultured striatal neurons

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Brain-derived neurotrophic factor (BDNF) is a potent trophic factor for striatal cells that promotes survival and/or differentiation of GABAergic neurons in vitro. In the present study, we show that the stimulation of cultured striatal cells with BDNF increased the phosphorylation of Akt and p42/p44. This effect was specifically blocked by inhibitors of phosphatidylinositol 3-kinase (PI3-K) (LY294002 and wortmannin) or p42/p44 mitogen-activated protein (MAP) kinase (PD98059 and U0126) pathways. BDNF treatment induced an increase in the number of calbindin-positive neurons but not in the number of GABAergic or total cells. Furthermore, BDNF increased the degree of dendritic arborization, soma area and axon length of striatal neurons. However, PD98059 was more effective blocking BDNF effects on calbindin- than on GABA-positive neurons, whereas LY294002 inhibited morphological differentiation in both neuronal populations. Moreover, BDNF induced neuronal survival only through the activation of the PI3-K pathway.

Introduction

The striatum is the major afferent component of the basal ganglia where it participates in the regulation of motor and cognitive functions (Gerfen, 1992). The predominant neuronal type in the striatum is the medium-sized spiny neuron (MSN) which contains GABA as a primary neurotransmitter. Although these neurons are morphologically homogeneous, they can be subdivided depending on receptor and neurotransmitter expression, patch or matrix localization and projecting areas (Graybiel, 1990). The calcium binding protein, calbindin, is a specific marker for MSN. Calbindin transiently labels patch neurons at birth but later becomes a marker for a large subset of matrix neurons (Liu and Graybiel, 1992). Because GABA expression in MSN is earlier than that of calbindin (Lauder et al., 1986), the acquisition of the calbindin phenotype can be considered as a late stage of maturation of striatal GABAergic neurons.

Neurotrophic factors participate in the development, establishment and maintenance of striatal connectivity. The neurotrophin brain-derived neurotrophic factor (BDNF) regulates striatal neuron survival (Alcantara et al., 1997) and maturation (Jones et al., 1994) during development. Furthermore, at early stages as well as at the later ones, the BDNF receptor, TrkB, is localized in MSN (Merlio et al., 1992). The source of striatal BDNF can be intrinsic, because BDNF mRNA and protein are detected in the striatum (Canals et al., 1998; Checa et al., 2000; Katoh-Semba et al., 1997; Yurek et al., 1998), or extrinsic by anterograde transport from the cortex or substantia nigra (Altar et al., 1997). Biological effects of BDNF on striatal cells in vitro comprise the induction of GABAergic neuron survival and/or differentiation (Mizuno et al., 1994; Ventimiglia et al., 1995). In this context, BDNF regulates the expression of specific markers of MSN maturation such as DARPP-32 or calbindin proteins (Ivkovic and Ehrlich, 1999; Ivkovic et al., 1997). BDNF is also a neuroprotective factor for excitotoxic-injured MSN (Bemelmans et al., 1999; Gratacos et al., 2001a; Perez-Navarro et al., 1999, 2000) or against hypoxic–ischemic insults (Han and Holtzman, 2000; Larsson et al., 1999). As these striatal neurons degenerate in Huntington's disease, knowledge of intracellular signaling pathways mediating BDNF regulation of MSN phenotype and/or survival could help to develop new therapeutical strategies for this neurodegenerative disorder.

Binding of BDNF to its receptor, TrkB, activates multiple intracellular signal transduction pathways, such as p42/p44 mitogen-activated protein kinase (MAP) kinase, phosphatidylinositol 3-kinase (PI3-K) and phospholipase C-γ1 (PLC-γ1) pathways (Chao, 2003; Patapoutian and Reichardt, 2001). Signaling through PI3-K or p42/p44 MAP kinase pathways leads to different trophic effects. The most widespread paradigm is that the PI3-K pathway is involved in survival (Hetman et al., 1999; Vaillant et al., 1999), while the p42/p44 MAP kinase pathway has been related to neuronal differentiation (Perron and Bixby, 1999). However, this pathway could also be involved in cell survival (Feng et al., 1999;

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Parrizas et al., 1997). In the striatum, it has been described that the regulation of DARPP-32 expression in MSN in vitro requires the activation of the PI3-K pathway (Stroppolo et al., 2001), but the intracellular pathways that are responsible for the biological effects of BDNF are not yet well understood.

In the present study, we examine the intracellular pathways activated by BDNF in MSN in vitro and whether these pathways contribute to BDNF-induced striatal neuron survival and GABAergic neuron maturation to a calbindin phenotype.

Results

BDNF induces the activation of PI3-K and p42/p44 MAP kinase pathways

To test whether BDNF was able to activate PI3-K and p42/p44 MAP kinase pathways in striatal cultures, cells were stimulated with BDNF (10 ng/ml) at 3 days in vivo (DIV), and the phosphorylation of Akt and p42/p44 was examined by Western blot at different time points. Our results show that Akt (Fig. 1A) and p42/p44 (Fig. 1B) were phosphorylated after BDNF treatment with a similar time course. Both proteins presented an enhancement of phosphorylation from 5 min to 1 h after BDNF treatment, whereas at 8 h were already decreased (Figs. 1A and 1B). However, the levels of phospho-p42/p44, but not phospho-Akt were still over control levels after 24 h or even 7 days.

Cultures were then treated for 1 h, before the addition of BDNF, with selective inhibitors of PI3-K (LY294002 or wortmannin) or of p42/p44 MAP kinase (PD98059 or U0126) pathways to determine their efficacy in blocking BDNF effects. Treatment with LY294002 (25–50 μM) or wortmannin (50–100 nM) prevented the phosphorylation of Akt induced by BDNF (Fig. 2A). Similarly, the addition of PD98059 (25–50 μM) or U0126 (5–10 μM) blocked the increase in phospho-p42/p44 produced by this neurotrophin (Fig. 2B). Furthermore, the inhibition of PI3-K pathway did not modify BDNF-induced p42/p44 phosphorylation (Fig. 2C), indicating that there was not an interaction between these two pathways. Therefore, BDNF is able to activate independently both PI3K and p42/p44 MAP kinase pathways in cultured striatal neurons.

BDNF specifically induces the maturation of striatal GABAergic neurons to a calbindin phenotype through PI3-K and p42/p44 MAP kinase pathways

We next investigated the putative neurotrophic effect of BDNF (10 ng/ml) on 3DIV cultured striatal neurons. Hence, the number of total cells, calbindin-positive neurons and GABAergic neurons were analyzed at different time points. In control conditions, the number of total cells was similar at different DIV examined (4DIV: 26,024 ± 1670 cells/cm^2; 10DIV: 27,177 ± 1088 cells/cm^2) and did not change when BDNF was added to the cultures (4DIV: 26,582 ± 2908 cells/cm^2; 10DIV: 28,163 ± 449 cells/cm^2). Similarly, the number of calbindin-positive neurons was unchanged after 7 days in control cultures (Fig. 3A). In contrast, when BDNF was added to the cultured neurons at 3DIV, a gradual increase was observed in the number of calbindin-positive neurons, reaching the maximal effect at 7DIV (442% from control values; Fig. 3A). Moreover, BDNF had to be maintained in the medium of striatal cultures to be effective, because when the neurotrophin was added at 3DIV and removed 24 h later, the number of calbindin-positive cells only increased to 163 ± 11% at 10DIV. These values were similar to those obtained after BDNF treatment between 3DIV and 4DIV.

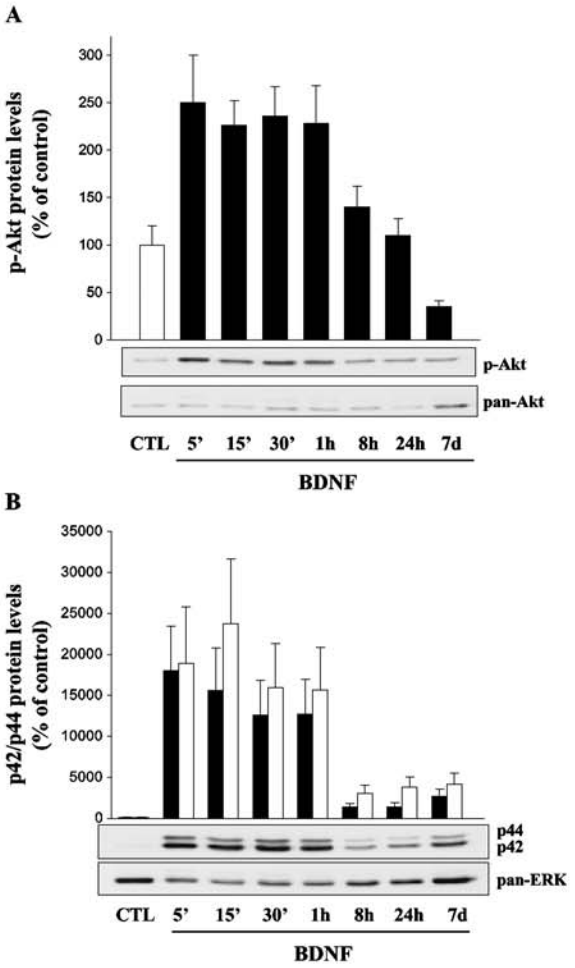
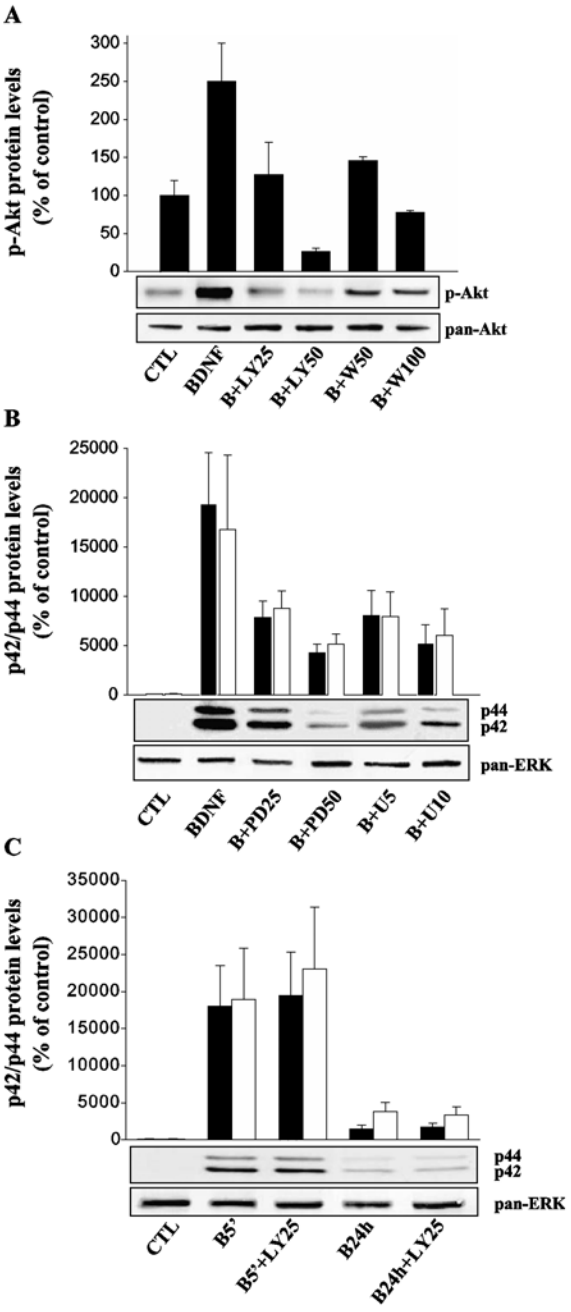


Fig. 1. BDNF induces the activation of PI3-K and p42/p44 MAP kinase pathways. BDNF (10 ng/ml) was added to the cultures and phosphorylation of Akt and p42/p44 was examined by Western blot at different time points. Figures show the quantification of the blots with results expressed as percentages of control, whereas autoradiograms were obtained from representative experiments. (A) Bars showing phospho-Akt protein levels ($n = 3$). (B) Bars showing phospho-p42/p44 protein levels ($n = 3$). p44: filled bars; p42: open bars.

Because both PI3-K and p42/p44 MAP kinase pathways were activated by BDNF, we examined whether these pathways were necessary for BDNF-induced increase in the number of calbindin-positive neurons. Cultures were treated at 3DIV with BDNF (10 ng/ml) with or without the addition of LY294002 (25 μM) or PD98059 (50 μM) and calbindin-positive neurons were counted at 10DIV. Fig. 3B shows that the effect of BDNF on calbindin-positive neurons was blocked by both inhibitors.

Calbindin-positive neurons are a subpopulation of GABAergic neurons. Thus, GABA-positive neurons were also examined in the same conditions. In our control culture conditions, calbindin-positive neurons represented only 8% of the GABA-positive

neurons. This percentage was increased to 40% in the presence of BDNF, but in contrast to the effect observed on calbindin-positive neurons, the number of GABA-positive neurons was not changed by BDNF treatment (control: 16,252 ± 2410 cells/cm^2; BDNF: 17,662 ± 1150 cells/cm^2). The presence of the inhibitors LY294002 (25 μM) and PD98059 (50 μM) did not modify significantly the number of GABA-positive neurons (LY294002: 88 ± 7%; PD98059: 111 ± 6% respect to control values), although higher doses were toxic (data not shown).



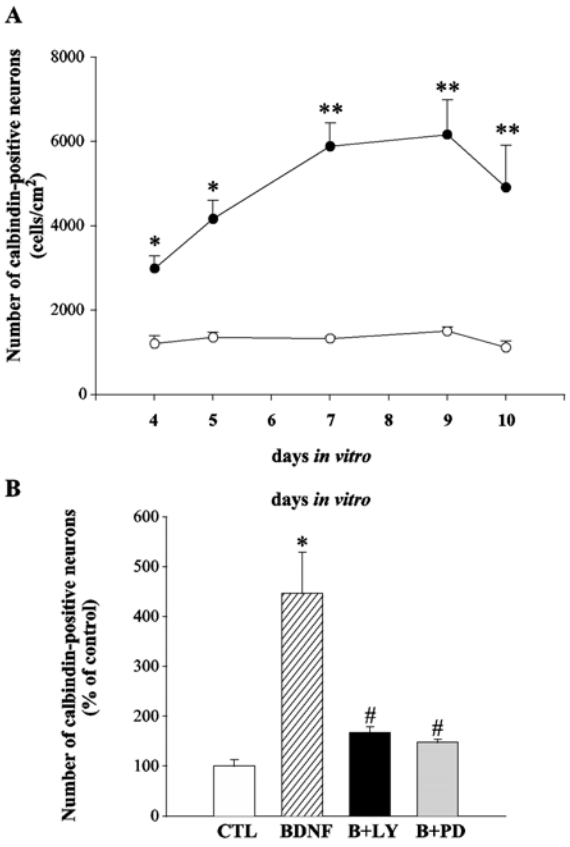


Fig. 3. BDNF specifically increases the number of calbindin-positive striatal neurons. (A) Graph showing the number of calbindin-positive neurons at different time points. Control: open circles; BDNF 10 ng/ml: filled circles. BDNF induced a progressive increase in the number of calbindin-positive neurons, reaching the maximal effect at 7DIV. (B) Graph showing the number of calbindin-positive neurons at 10DIV with results expressed as a percentage of control values (CTL). Treatment with BDNF increased the number of calbindin-positive neurons (BDNF). This effect was blocked by the addition of LY294002 25 μM (B + LY) or PD98059 50 μM (B + PD). (A and B) Results are the mean ± SEM of six determinations from three different experiments. $*P < 0.05$ and $**P < 0.001$ compared to control values. One-way analysis of variance (ANOVA) followed by the Scheffé post hoc test.

To rule out a BDNF-induced proliferative process, nestin-positive cells were counted 48 h after BDNF (10 ng/ml) addition to cultures at 3DIV. No significant changes were observed between

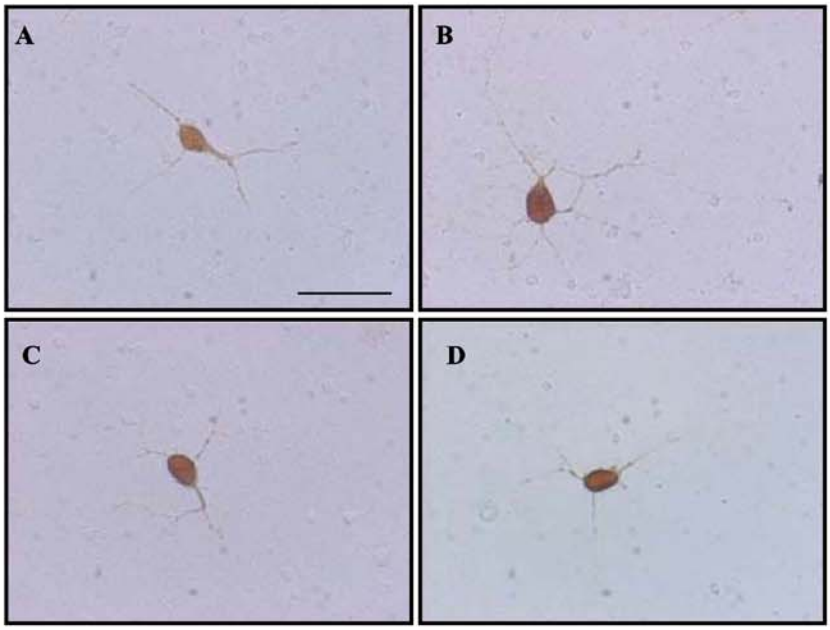
Fig. 2. PI3-K and p42/p44 MAP kinase pathways inhibitors blocked BDNF effects. Figures show the quantification of the blots with results expressed as percentages of control. Autoradiograms were obtained from representative experiments. (A) Bars showing phospho-Akt protein levels as assessed by Western blot analysis ($n = 3$). BDNF (10 ng/ml) induces an increase in phospho-Akt that is selectively blocked by the addition of LY294002 25 μM (B + LY25) or 50 μM (B + LY50); wortmannin 50 nM (B + W50) or 100 nM (B + W100). (B) Bars showing phospho-p42/p44 protein levels as assessed by Western blot analysis ($n = 3$). BDNF induces the phosphorylation of p42/p44 that is selectively blocked by the addition of PD98059 25 μM (B + PD25) or 50 μM (B + PD50); U0126 5 μM (B + U5) or 10 μM (B + U10). (C) Bars showing phospho-p42/p44 after addition of BDNF plus LY294002 25 μM during 5 min (B5′ + LY25) or 24 h (B24h + LY25). p-44: filled bars; p42: open bars.

control or BDNF-treated cells (control: 1011 ± 72 cells/cm^2; BDNF: 1072 ± 101 cells/cm^2).

BDNF promotes the arborization of GABA- and calbindin-positive striatal neurons through PI3-K and p42/p44 MAP kinase pathways

Another step in neural differentiation is neurite growth. Thus, we examined whether BDNF was involved in the degree of arborization of GABA- and calbindin-positive striatal neurons. BDNF (10 ng/ml) was added to the cultured neurons at 3DIV and morphological parameters were analyzed at 10DIV. In contrast to that observed on the number of neurons, BDNF induced an increase in the total and soma areas, perimeter, axon length and degree of arborization of both GABA- and calbindin-positive neurons (Figs. 4 and 5).

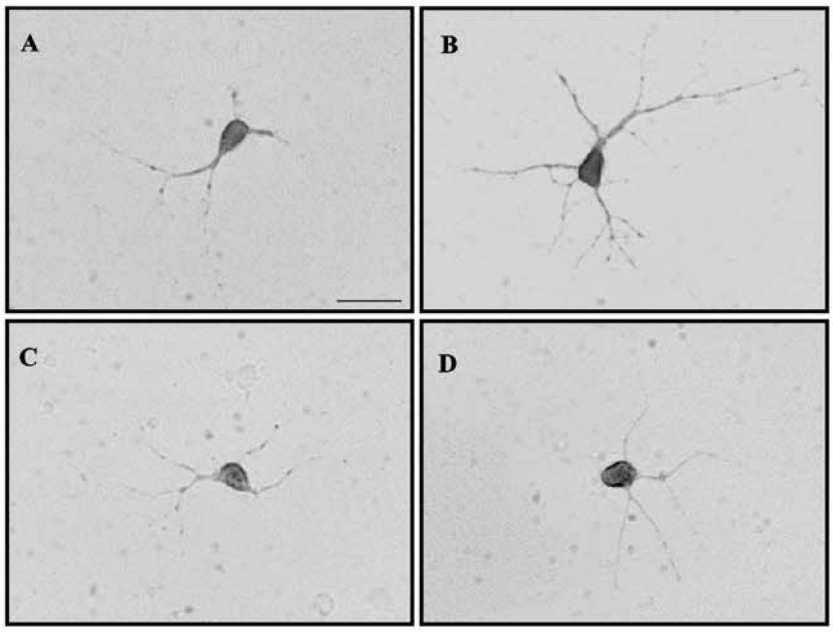
The involvement of PI3-K and p42/p44 MAP kinase pathways in these effects was also studied. LY294002 (25 μM) or PD98059 (50 μM) were added to 3DIV cultures together with BDNF, and morphological parameters were analyzed 7 days later. Our results show that the presence of LY294002 or PD98059 differentially blocked BDNF effects depending on the population examined. Hence, LY294002 had a similar effect on GABA- and calbindin-positive neurons, whereas PD98059 was more effective on calbindin-positive neurons than GABAergic cells (Figs. 4 and 5). The treatment with LY294002 (25 μM) or PD98059 (50 μM) alone induced a decrease in all the morphological parameters



E Quantitative analysis of the differentiation induced by BDNF on calbindin-positive striatal neurons.

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
Control	246 ± 20	326 ± 6	118 ± 10	65 ± 8	36 ± 2
BDNF	440± 19*	670± 36*	150± 15*	83± 11*	84± 5*
BDNF+LY	236 ± 7#	346 ± 4#	103 ± 6#	57 ± 2#	41 ± 2#
BDNF+PD	289 ± 37#	408 ± 33#*	127 ± 14 #	63 ± 3#	47 ± 2#

Fig. 4. BDNF promotes calbindin-positive neurons differentiation through PI3-K and ERK MAP kinase pathways. Calbindin immunocytochemistry was performed at 10DIV. Photomicrographs show calbindin-positive neurons from striatal cultures treated with (A) vehicle, (B) BDNF (10 ng/ml), (C) BDNF + LY294002 25 μM or (D) BDNF + PD98059 50 μM. Scale bar = 28 μm. (E) Quantitative analysis of BDNF effects on striatal calbindin-positive cells morphology and involvement of PI3-K and ERK MAP kinase pathways. For each parameter and condition examined, 60 neurons were analyzed in three different experiments. Results are expressed as the mean ± SEM. $*P < 0.05$ compared to control values, $\#P < 0.05$ compared with BDNF values. ANOVA followed by the Scheffé post hoc test.



E **Quantitative analysis of the differentiation induced by BDNF on GABA-positive striatal neurons.**

	Total area (μm^2)	Perimeter (μm)	Soma area (μm^2)	Axon length (μm)	Degree of arborization
Control	203 ± 10	292 ± 27	100 ± 1	55 ± 6	35 ± 5
BDNF	470 ± 75*	771 ± 87*	147 ± 22*	86 ± 9*	104 ± 8*
BDNF+LY	241 ± 15#	359 ± 6#	101 ± 9#	64 ± 8#	44 ± 1#
BDNF+PD	363 ± 59#*	547 ± 56*	128 ± 9*	68 ± 3#	68 ± 4# *

Fig. 5. BDNF promotes the arborization of GABA-positive neurons through PI3-K and ERK MAP kinase pathways. GABA immunocytochemistry was performed at 10DIV. Photomicrographs show GABA-positive neurons from striatal cultures treated with either (A) vehicle, (B) BDNF (10 ng/ml), (C) BDNF + LY294002 25 μM or (D) BDNF + PD98059 50 μM. Scale bar = 28 μm. (E) Quantitative analysis of BDNF effects on striatal GABA-positive cells morphology and involvement of PI3-K and ERK MAP kinase pathways. For each parameter and condition examined, 60 neurons were analyzed in three experiments. Results are expressed as the mean ± SEM. $*P < 0.05$ compared to control values, $\#P < 0.05$ compared to BDNF values. ANOVA followed by the Scheffé post

analyzed that never reached more than 20% of reduction (data not shown).

BDNF mediates survival through PI3-K pathway

The involvement of PI3-K and p42/44 MAP kinase pathways on the survival of striatal neurons was also examined. BDNF (10 ng/ml) was added at 3DIV and 2 days later, dying neurons with pyknotic or fragmented nuclei were counted using DAPI staining. Our results show that BDNF reduced the number of dying neurons by 31% (Fig. 6A). However, the number of dying neurons in our culture conditions was only 4% of the total number of neurons. This could explain the lack of changes in the total number of cells after BDNF treatment. The involvement of PI3-K and p42/p44 MAP kinase pathways was also examined using the inhibitors LY294002 and PD98059, respectively. LY294002, but not PD98059, blocked the effect of BDNF on neuronal survival (Fig. 6A), suggesting that the PI3-K pathway mediates BDNF-induced neuronal survival. However, PD98059 (50 μM) did not blocked completely the phosphorylation of p42/p44 (Fig. 2B), and this remaining activity may also produce some effects. To discard this possibility, we found a low dose of BDNF (0.5 ng/ml), without biological effects, but with similar levels of phosphorylation of p42/44 that were induced by BDNF (10 ng/ml) plus PD98059 (50 μM) (Fig. 6B). Furthermore, the addition of PD 98059 plus LY294002 together with BDNF (10 ng/ml) did not increase the

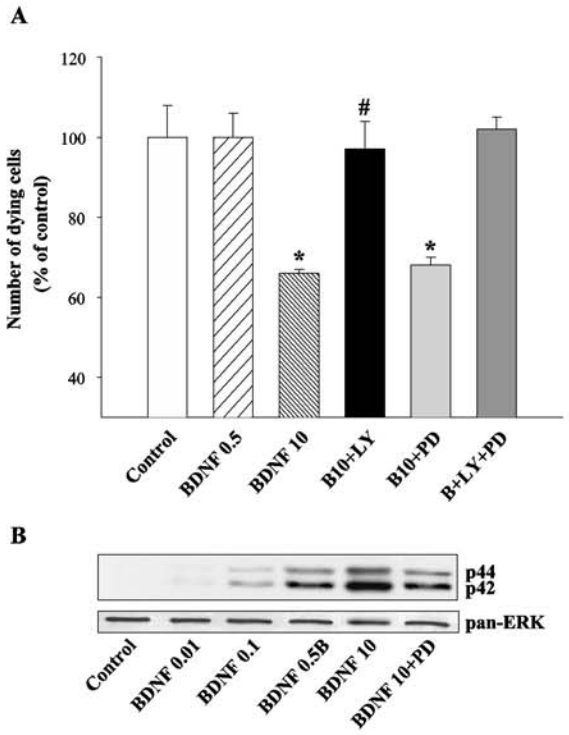


Fig. 6. BDNF promotes survival of striatal neurons through the PI3-K pathway. (A) Bars showing the number of dying cells, expressed as a percentage of control, after addition of BDNF 0.5 ng/ml (BDNF 0.5), BDNF 10 ng/ml (BDNF 10), BDNF (10 ng/ml) plus 25 μM LY294002 (B10 + LY), BDNF (10 ng/ml) plus 50 μM PD98059 or BDNF (10 ng/ml) plus both inhibitors (B + LY + PD). Values indicate the mean ± SEM of three independent experiments, each comprising six wells. $*P < 0.05$ compared with control values, $\#P < 0.05$ compared with BDNF values. ANOVA followed by the Scheffé post hoc test. (B) Autoradiogram showing p42/p44 phosphorylation 5 min after addition of 0.01 ng/ml BDNF (BDNF 0.01), 0.1 ng/ml BDNF (BDNF 0.1), 0.5 ng/ml BDNF (BDNF 0.05), 10 ng/ml BDNF (BDNF 10) or 10 ng/ml BDNF plus 50 μM PD98059 (BDNF 10 + PD).

number of dying neurons compared to control values (Fig. 6A). These results confirm that the activation of p42/p44 MAP kinase pathway is not required to induce BDNF-promoting survival effects in striatal neurons.

Discussion

Present results show that both PI3-K and p42/p44 MAP kinase pathways are involved in the effect of BDNF in the maturation of striatal GABAergic neurons in vitro. BDNF treatment favors the acquisition of the calbindin phenotype, because it increased the number of calbindin-positive cells without affecting GABAergic neurons and total cell number. This effect was blocked when specific inhibitors of the PI3-K (LY294002) or of the p42/p44 MAP kinase (PD98059) pathways were added to the cultures. Calbindin-positive neurons and also GABAergic cells increased the degree of dendritic arborization, soma area and axon length in the presence of BDNF. Our data indicate that the activation of PI3-K and p42/p44 MAP kinase pathways is differentially required for BDNF-mediated effects on the differentiation of striatal neurons depending on the neuronal population studied. Furthermore, BDNF also induced striatal neuron survival by a mechanism only involving PI3-K pathway activation.

The binding of neurotrophic factors to their receptors can activate different intracellular pathways (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Here, we show that BDNF activates both PI3-K and p42/p44 MAP kinase pathways on striatal neurons as shown by transient increased levels of phosphorylated Akt and p42/p44. The peak of phosphorylation of both proteins was very similar between 5 min and 1 h. However, after 8 h of BDNF treatment, phospho-Akt levels almost reached control values, whereas the phosphorylation of p42/p44 was still over control values at 24 h and even at 7 days. This could explain why the presence of BDNF in the culture medium is required to reach the maximal biological effects. Similarly, a persistent activation of TrkA receptors is needed to maintain neuronally differentiated PC12 cells (Chang et al., 2003). The activation induced by BDNF was blocked by the addition of specific inhibitors such as LY294002 and wortmannin, for the PI3-K pathway, or PD98059 and U0126 for the p42/p44 MAP kinase pathway. No effect was observed of inhibitors of the p42/p44 MAP kinase pathway in phospho-Akt levels, nor of inhibitors of PI3-K pathway in the levels of p42/p44, suggesting an independent activation of PI3-K and p42/p44 MAP kinase pathways during TrkB stimulation. Similarly, TrkB receptor-mediated activation of p42/p44 cascade has been shown to occur independently of PI3-K activity in several cell systems (Kaplan and Miller, 2000) as well as in striatal primary cultures (Perkinton et al., 2002). However, cross talk between these two intracellular pathways has been also shown in striatal cultures in response to other types of treatment, like NMDA or KA receptor stimulation, which induces a PI3-K-dependent p42/p44 activation (Fuller et al., 2001; Perkinton et al., 2002).

Our results show that BDNF contributes to the maturation of striatal neurons and helps them to acquire the calbindin phenotype. The treatment of striatal cultures with BDNF increased by 4-fold the number of calbindin-positive cells, according to previous studies (Ventimiglia et al., 1995). This effect was mostly due to an increase in calbindin expression because the number of GABAergic neurons, total cells and nestin-positive cells was not modified by the addition of BDNF. In agreement with our results, calbindin expression is reduced in the BDNF knockout animal, but GABAergic cells are present in normal density, suggesting that this factor is important in regulating neuronal differentiation (Jones et al., 1994). On the other hand, several in vivo and in vitro studies show the involvement of BDNF in the regulation of the expression of other specific markers for MSN, such as DARPP-32 and ARPP-32 (Ivkovic and Ehrlich, 1999; Ivkovic et al., 1997). Our results showed that the intracellular signaling cascades required by BDNF to induce the acquisition of the calbindin phenotype are the PI3-K and the p42/p44 MAP kinase pathway, because treatment with LY294002 or PD98059 blocked its effects on calbindin-positive neurons. Accordingly, the PI3-K pathway has been shown to regulate BDNF-induced DARPP-32 expression in MSN (Stroppolo et al., 2001).

The effects of BDNF on striatal neurons maturation were not limited to the induction of the calbindin phenotype because it also increased the degree of arborization, axon length and the soma size on both GABA- and calbindin-positive neurons. The activation of both PI3-K and p42/p44 MAP kinase pathways were necessary for BDNF to achieve these effects. Previous studies using other cellular systems have also shown an involvement of both pathways in

differentiation processes such as neurite extension (Atwal et al., 2000; Encinas et al., 1999; Pang et al., 1995; Qiu and Green, 1991; Sanchez et al., 2001; Tsui-Pierchala et al., 2000; York et al., 2000) and axon branching or elongation (Markus et al., 2002). However, in the present culture system, these pathways may have different roles, depending on the neuronal population examined. The inhibitor of the PI-3K, LY294002, was very effective in blocking BDNF-mediated effects on both neuronal populations. In contrast, the inhibition of BDNF effects observed after treatment with PD98059 was more pronounced on calbindin- than on GABA-positive neurons, suggesting that the activation of the p42/p44 MAP kinase is essential to induce late stages of neuronal maturation. Interestingly, the activation of the p42/p44 signaling system has been shown to be involved in the control of mature neuronal functions such as the formation of novel dendritic spines associated with synaptic plasticity (Goldin and Segal, 2003), and in learning and memory (Mazzucchelli et al., 2002).

BDNF can also regulate cell survival in vitro (Nakao et al., 1995; Ventimiglia et al., 1995) and in vivo (Perez-Navarro et al., 2000). In the present culture conditions, there was a small number of dying cells as shown by DAPI staining, and some of these cells were calbindin-positive neurons. Treatment with BDNF reduced the number of dying cells by 30% but no changes were detected in the number of total cells as apoptotic cells were less than 4% of total cells. In contrast to the BDNF effects on differentiation and maturation, only the addition of LY294002 blocked BDNF-promoting survival effects showing that BDNF depends on the activation of the PI3-K to exert this effect. Similarly, the activation of the p42/p44 MAP kinase pathway is not necessary for survival of sympathetic neurons (Creedon et al., 1996; Virdee and Tolkovsky, 1996), hippocampal pyramidal neurons (Marsh and Palfrey, 1996) and chicken spinal motor neurons (Dolcet et al., 1999; Soler et al., 1998).

In conclusion, our data demonstrate that the activation of PI3-K and p42/p44 MAP kinase pathways is differentially involved in BDNF effects on cultured striatal neurons. The activation of both pathways mediates BDNF effects on the differentiation and maturation of striatal GABAergic neurons, whereas only the PI3-K plays a pivotal role in BDNF-promoting survival effects.

Experimental methods

Cell culture

Animal handling procedures were approved by the Local Committee (99/1 University of Barcelona) and the Generalitat de Catalunya (1094/99), in accordance with the Directive 86/609/EU of the European Commission. Certified time-pregnant Sprague–Dawley dams (Iberfauna, Spain) were deeply anesthetized on gestational day 19, and fetuses were rapidly removed from the uterus, as previously described (Gratacos et al., 2001b). Cells were plated at a density of 50,000 cells/cm^2 onto 24-well plates or 100-mm culture dishes, which were precoated with 0.1 mg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO, USA), for morphological or Western blot analysis, respectively. Eagle's minimum essential medium (MEM; Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B-27 (Gibco-BRL) was used to grow the cells in serum-free conditions. To study the activation of PI3-K and p42/p44 MAP kinase pathways, medium was removed on 2DIV and replaced by N2-supplemented medium to deprive cells for 24 h. Then, BDNF (10 ng/ml; Peprotech EC Ltd., London, UK) was added to the cultures and Akt and p42/p44 phosphorylation examined at different DIV. In other set of experiments, cultures were treated with distinct inhibitors, such as LY294002 (25 or 50 μM; Biomol Research Laboratories, USA), PD98059 (25 or 50 μM; Calbiochem, San Diego, CA), wortmannin (50 or 100 nM; Calbiochem) or U0126 (5 or 10 μM; Calbiochem). All these inhibitors were dissolved with N2-supplemented medium with bovine serum albumin (BSA, 6.6 mg/ml; Sigma), and added 1 h before BDNF treatment. For morphological analysis, MEM supplemented with B-27 was used to grow the cells and at 3DIV BDNF (10 ng/ml) was added alone or co-incubated with LY294002 or PD98059, and 6.6 mg/ml BSA was also added to the medium. Plated cell cultures were maintained in an incubator with 5% CO_2 at 37°C.

Western blot analysis

After BDNF exposure, cells were rinsed rapidly in ice-cold phosphate-buffered saline (PBS), and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 10 mM EGTA and 150 mM NaCl) plus protease inhibitors (2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM orthovanadate). For detection of phosphorylated forms of p42/p44 and Akt (S473), cell lysates were resolved in 8% denaturing polyacrylamide gel using the Mini-protean system (BioRad). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore), washed twice in Tris-Buffered saline containing 0.1% Tween-20 (TBS-T) and incubated for 1 h with 5% BSA and 5% fat-free dry milk in TBS-T. Membranes were then incubated overnight at 4°C with antibodies against phospho-p42/p44 (1:10,000; Cell Signaling Technology, Beverly, MA) or phospho-Akt (1:2000; Cell Signaling Technology). Next day, after two rinses with TBS-T, membranes were incubated for 1 h at room temperature (r.t.) with horseradish peroxidase-conjugated anti-rabbit antibody (1:2000; Promega), and the reaction was finally visualized with the Super Signal Chemiluminescent Substrate (Pierce, Tattenhall, UK). To standardise total protein content in each lane, membranes were incubated for 1 h at r.t. with a mouse monoclonal antibody against panERK (1:5000; BD Transduction Laboratories) or against panAkt (1:2000; Cell Signaling Technology). After two rinses with TBS-T, membranes were incubated for 1 h at r.t. with horseradish peroxidase-conjugated anti-mouse antibody (1:2000). The reaction was also visualized with the Super Signal Chemiluminescent Substrate. Western blot replicates were scanned and quantified using the Phoretix 1D Gel Analysis (Phoretix International Ltd., Newcastle, UK).

Immunocytochemistry

Striatal cultures were fixed with 4% paraformaldehyde for 1 h at r.t., followed by three rinses in PBS. Cells were then preincubated for 15 min with PBS containing 0.3% Triton X-100 (Sigma) and 30% normal horse serum (Gibco-BRL) at r.t. Cultures were then incubated overnight at 4°C with antibodies directed against calbindin$_{D28K}$ (1:10,000; Swant), GABA (1:1000; Sigma) or nestin (1:40; S. Hockfield, Developmental Studies Hybridoma Bank, Iowa City, IA) diluted in PBS containing 0.3% Triton X-100 and 5% normal horse serum. Cells were then incubated in biotinylated secondary antibodies followed by the Pierce ABC Kit and then reacted with diaminobenzidine.

Detection of cell death

Detection of dying neurons at 5DIV was performed 2 days after treatment with BDNF (10 ng/ml) with or without the presence of inhibitors (25 μM LY294002 or 50 μM PD98059). Cells were fixed with 4% paraformaldehyde for 1 h at r.t., followed by three rinses in PBS. Neurons were incubated with DAPI (1:100; Sigma) for 5 min and then rinsed twice with PBS.

Quantitative analysis of cell cultures

Total cell number, GABAergic neurons, calbindin-positive neurons and dying cells stained by DAPI were counted within 20 fields at 200×. Results are given as number of cells/cm^2 or percentage of control cultures, analyzing four to six wells per condition from three different experiments. Morphological parameters were assessed using a PC-Image analysis system from Foster Findlay on a computer attached to an Olympus microscope. GABA and calbindin-positive neurons (60 per condition) were randomly chosen and traced in a phase-contrast image using the mouse hook up. Total and soma area, perimeter and degree of arborization (Perimeter^2/4πArea) were determined as described by Fujita et al. (1996). Axon length was also measured, considering the axon as the longest emerging neurite from the soma, as previously described (Gratacos et al., 2001b).

Statistical significance was assessed by ANOVA followed by the Scheffé post hoc test.

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